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Optimization of medium components using orthogonal arrays for γ-Linolenic acid production by *Spirulina platensis*

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Abstract−This work describes the medium optimization of γ-Linolenic acid (GLA) production by *Spirulina platensis* using one-factor and orthogonal array design methods. In the one-factor experiments, $NaHCO_3$ (9 mg L^{-1}), $NaNO_3$ (13.5 mg L^{-1}) and $MgSO_4 \cdot 7H_2O$ (11.85 mg L^{-1}) proved to be the best components for GLA production. The optimal pH for GLA production by the alga was 9.2. Based on the delta values, $NaHCO_3$ showed the greatest effect on the GLA production of the various factors tested, followed in decreasing order by $MgSO_4 \cdot 7H_2O$, $NaNO_3$ and K_2SO_4. The maximum GLA yield obtained was 19.2 mgL^{-1} in the presence of optimum concentrations of $NaHCO_3$ (20 g L^{-1}), $NaNO_3$ (3 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}) and K_2SO_4 (1.5 g L^{-1}). Because of the slow growth rate of the algae, the practice of robust orthogonal array methods during the optimization of medium components can result in the production of an optimal biomass and a higher GLA yield for nutraceutical applications.

Keywords: γ-Linolenic Acid, Optimization, Orthogonal Design, *Spirulina platensis*

INTRODUCTION

GLA (γ-Linolenic acid), an important omega-6 fatty acid, is an antecedent in the production of prostaglandin E1 and other essential fatty acids. It has a wide variety of applications in pharmaceuticals, feed, and functional foods. In addition, GLA plays an active role in the treatment of various disorders, such as malignant glioma [1], rheumatoid arthritis [2], hypertension [3], diabetic neuropathy [4] and atopic eczema [5].

Although GLA has been typically obtained from plant oils, the concentration (w/w) of this fatty acid in plant oils is low, as indicated by the following examples: primrose oil, 7-10%; borage seed oil, 21-25%; and black current seed oil, 15-20% [6]. Novel microbial sources, such as fungi and algae, have become alternatives for GLA production. A small number of fungi, such as *Mucor* spp. and *Mortierella* spp., are potential producers of GLA. However, the presence of the alpha isomer of GLA as a contaminant in GLA obtained from fungal sources incurs additional costs in scale-up, purification and production.

The blue-green alga *Spirulina platensis* is a well-known algal source of γ-Linolenic acid [7]. The average lipid content observed in this alga was 4-7%, and GLA accounts for a maximum of 31% of the total fatty acids [8]. The highest reported GLA yield from this alga was 1% (w/w), based on dry weight and 20.8 mg L^{-1}, based on volume [9].

GLA from this alga is particularly attractive because of its potential to incorporate the whole source as a food. In addition, this alga can be easily cultivated using natural sunlight, air and simple inorganic nutrients. This alga is typically grown in either a bicarbonate- or a carbonate-enriched medium [10]. The conventional nitrogen source for this algal species is $NaNO_3$ [11-13]. However, alternative nitrogen sources include KNO_3, urea, and NH_4NO_3. In addition, several mineral sources influence algal growth. Magnesium is an essential macro element for the growth of this alga [14].

In media-optimization studies, a relatively simple approach involves varying one factor (the medium component) of interest while keeping other factors constant (the one-factor method). However, this is laborious when investigations involve determining the responses of multiple factors and their combinations.

The conventional full factorial design involves substantial effort and time but achieves less reliable results. As an alternative to the full factorial design, we employed the Taguchi method [15,16]. The statistical design is an orthogonal array, which studies the responses of controlling factors at various levels. The design minimizes variation while the response is studied. The strength of an orthogonal array relies on its ability to show the response of each factor level independently without masking the response of other factor levels in a given run.

Although much attention has been paid to studying the effects of medium components (nitrogen in particular) on algal growth and biomass production [13], few studies [9] have looked at the role of nitrogen in essential fatty acid yields, its specific content, or its composition in total fatty acids (TFA). Considering the inherently slower growth rate of the alga compared with other organisms, the cultiva-

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tion of the alga for desired metabolites (proteins, lipids and pigments) requires a well-balanced medium with respect to carbon, nitrogen and mineral concentrations.

To date, there are only a few studies on the alga regarding the effect of medium components on GLA production [9,17]. Hence, the present work reports the optimization of GLA production by *Spirulina platensis* with respect to carbon, nitrogen, mineral salts and pH in the medium. The best sources were considered factors that affect GLA production. By using an orthogonal array, the effect of each factor was further evaluated in combination with other factors.

MATERIALS AND METHODS

1. Microalgae and Media Composition

Spirulina platensis ARM-740 was obtained from a culture collection of CFTRI (Mysore, India). All media optimization experiments were performed with the SOT production medium [6] with the following composition (g L^{-1}): $NaHCO_3$: 16.8, $NaNO_3$: 2.5; K_2SO_4: 1.0; NaCl: 1.0, $MgSO_4 \cdot 7H_2O$: 0.2; K_2HPO_4: 0.5 $CaCl_2 \cdot 2H_2O$: 0.04, $FeSO_4 \cdot 7H_2O$: 0.01, EDTA: 0.08 and A-5 trace elemental solution 1.0 mL.

The A5 trace elemental composition (g L^{-1}) was as follows: H_3BO_3: 2.86, $MnCl_2 \cdot 4H_2O$: 1.81, $ZnSO_4 \cdot 7H_2O$: 0.222, $Na_2MoO_4 \cdot 2H_2O$: 0.039, $CuSO_4 \cdot 5H_2O$: 0.079 and $Co(NO_3)_2 6H_2O$: 0.49.

2. Cultivation Method and Culture Conditions

In submerged culture studies, the cultures were grown for eight days in 500 mL conical Erlenmeyer flasks with 250 mL culture medium at 32±1 ºC. The pH of the medium was initially adjusted to 9.2 with alkali (6 M NaOH) and was further allowed to change freely during the experiments. Flasks were shaken manually thrice a day to optimize mixing conditions. A culture suspension comprising 10% of working volume was used to inoculate the experimental flasks. Continuous illumination was provided with four 18-Watt fluorescent lights; the distance of the light source was adjusted to give an average light intensity of 1,500 lux. Concentration of the algal biomass in the experimental setups was determined by the dry weight method [18]. The algal samples for the fatty acid analysis were filtered using Whatman No. 3 filter paper, the cell concentrate was lyophilized, and the freeze-dried cells were subjected to fatty acid analysis. All the values of dry weight, fatty acid yield, and its composition were observed as the mean values in duplicate experiments.

3. Transmethylation of Lipids and Fatty Acid Methyl Ester Analysis

The amount of γ-Linolenic acid in total lipids was estimated by the conversion of fatty acids to their respective ester forms. In the process, lipids were directly transmethylated by treating freeze-dried samples of the alga with methanol-acetyl chloride mixture according to the procedure in Cohen et al. [7]. Heptadecanoic acid was used as an internal standard for quantification. Fatty acid methyl ester analysis (FAME) was performed using gas chromatography (Thermo Scientific, 8610) assembled with a FID detector and a BPX-70 capillary column (30 m×0.32 mm IDX 0.25 μ). A 70% cyanopropyl polysilphenylene-siloxane stationary phase was used for the fatty acid analysis.

The identity of the individual peak was established by comparing the retention times of the peak with authentic standards (Sigma & Co., Germany). The carrier gas (nitrogen) flow was adjusted at 35 mL min^{-1}, and the fatty acid sample analysis was done in an isothermal mode by maintaining the column temperature at 180 ºC. The injection port and detector temperatures were maintained at 250 ºC.

4. Experimental Design

The current study focuses on optimizing GLA production using a robust statistical design. Taguchi's design method was used to study the effect of various factors known to show variations in response. The method at the initial level involves selecting various influential factors that affect the yield (response) of a product. The influential parameters are screened using the one-factor-at-a-time (OFAT) method, which studies the effect of a single independent factor (carbon, nitrogen, mineral salts and pH) by manipulating its concentration, while keeping other factors at fixed levels.

When the factors causing variation in response were identified, various concentration levels were assigned to the factors. Depending on the number of factors and their concentration levels, a statistical program (Minitab; version 16, Minitab Inc., PA, USA) was used to generate various possible orthogonal arrays. The Taguchi method is intended to study the effect of a single factor when present in combination with several other factors. Because this method is well balanced, each factor (media component) in the array was given equal importance and its influence was tested independently for all other factors. The method assumes a minimum interaction among factors during the orthogonal runs.

5. Experiments Using the One Factor Method

The OFAT method enabled screening the best possible factor by keeping other factors constant in the optimization experiments. The OFAT method was used in a series of experiments, which tested the effect of various carbon, nitrogen sources, mineral salts, and pH on the biomass, fatty acid composition (GLA/TFA ratio) and GLA production. The effects of three inorganic carbon sources, $NaHCO_3$, K_2CO_3 and Na_2CO_3, were studied independently by maintaining their concentration levels at 10 g L^{-1} in the medium. Nitrogen sources, including urea, ammonium sulfate, sodium nitrate, and potassium nitrate were tested at each of four different concentration levels: 0.01%, 0.05%, 0.1%, and 0.25% (w/v). In studies with mineral sources, the effects of $MgSO_4$, $7H_2O$, K_2SO_4 and K_2HPO_4 on algal growth and GLA production were compared by maintaining the salts at 6 mM concentration in the medium. Optimum pH for GLA production was determined by cultivating algae in a standard SOT medium with four initial pH conditions: 8.2, 9.2, 10.2, and 11.2.

6. Statistical Analysis

The GLA response used for the statistical analysis was the mean± standard deviation (SD) in at least three independent experiments. All statistical analyses were carried out using SPSS version 21.0 software (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to compare the significant level of variations in means between groups .Statistical values of $P<0.05$ were considered significant, which rejected the null hypothesis between groups.

RESULTS AND DISCUSSION

1. Effect of Inorganic Carbon Sources

Several researchers [11,12,19] have formulated the optimal nutrient medium for the growth of algae. In *Spirulina* cultivation, bicarbonate is the predominant source of carbon. In a few applications,

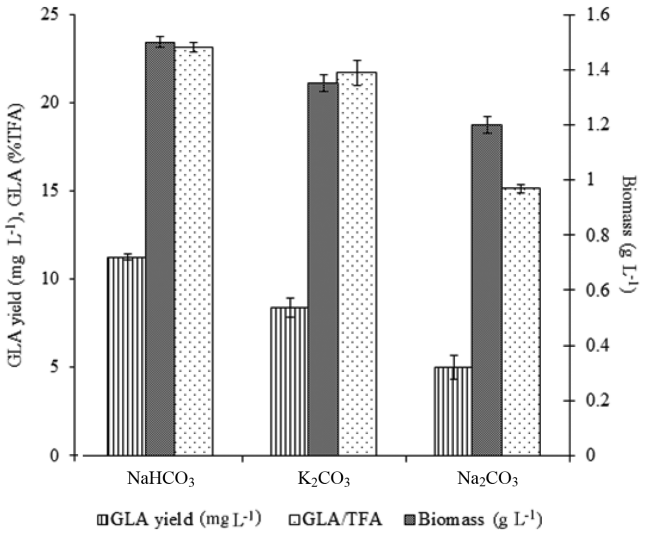

Fig. 1. Effect of Carbon sources on biomass, GLA yield and GLA composition.The GLA content (w/w) was found to be 0.49 and 0.33% with K_2CO_3 and Na_2CO_3 respectively as against 0.6% found with $NaHCO_3$. Carbon sources effecting GLA yield (w/v), which are statistically significant, were selected between groups. One way Annova : Between groups (F= 60.85; P<0.05).

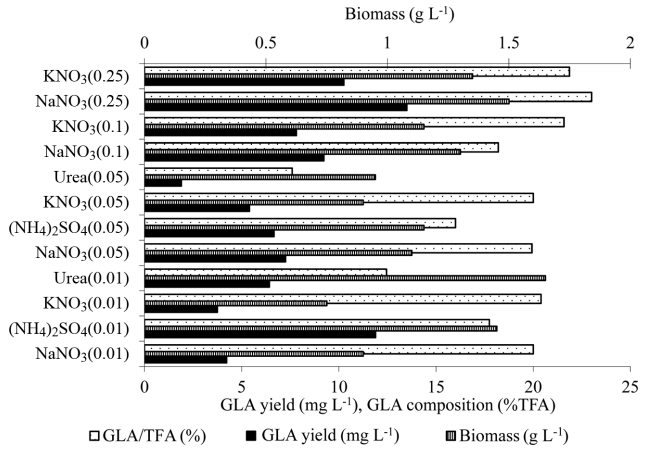

Fig. 2. Effect of various Nitrogen sources(w/v) on biomass and GLA production. Significant GLA accumulation (w/w) was found with 2.5 g L^{-1} $NaNO_3$ (0.9%) and 0.1 g L^{-1} $(NH_4)_2SO_4$ (0.82% GLA in dry cell weight). Urea at 0.01% (0.38% GLA in dry cell weight) and 0.05% (0.2% GLA in dry cell weight) showed poor GLA accumulation in biomass; One way annova was done to evaluate statistically significant GLA yield data for nitrogen groups. Between groups (0.01 w/v): F=70.52; P< 0.05; One way annova between groups (0.05 w/v): F=16.71, P<0.05.

a mixture of inorganic carbon sources can also be used to cultivate the species. One example is the cultivation of *Spirulina* in Schlösser's [20] medium, in which sodium bicarbonate and sodium carbonate are primary carbon sources. *Spirulina platensis* can use both organic and inorganic carbon sources; hence, it is of interest to observe the extent to which the growth and GLA are affected by the presence of various inorganic carbon sources.

The effect of the carbon source on GLA production is shown in Fig. 1: bicarbonate produced a maximum GLA yield (9.0 mg L^{-1}), biomass (1.5 g L^{-1}) and GLA/TFA ratio (18.5%). In addition, the results showed that alga could not utilize carbonate as efficiently as bicarbonate could; sodium carbonate was an ineffective carbon source, yielding relatively low GLA (4 mg L^{-1}). In the current study, GLA production was affected in the following order: $NaHCO_3$>K_2CO_3> $NaCO_3$.

The influence of $NaHCO_3$ on algal growth and fatty acid production was attributed to the dominance and availability of the bicarbonate ion at the functional pH. Although direct evidence is not available in the literature regarding the comparison of GLA yield of carbonate sources, the results can be correlated indirectly with the maximum growth and specific yield of GLA, which were attained in the bicarbonate-enriched medium. At higher alkalinity (pH>10.0), the bicarbonate exists as a carbonate. The growth and product formation at this pH level was attributed to the influence of carbonate on GLA production.

Several findings [21-23] showed the optimal growth of the biomass at pH: 9.0 (HCO_3^-), compared to high alkalinity (CO_3^{2-}). Although biomass concentration is an influential parameter on optimal GLA yield, with restrictions on maximum possible specific GLA yield, any carbon source promoting high biomass and moderate to high specific GLA yield is ideal for optimization. Bicarbonate is a widely accepted carbon source with a high specific GLA content. Hence, this carbon would be an attractive choice for further optimization experiments in orthogonal array runs.

2. Effect of Nitrogen Sources

While nitrate is generally considered a primary nitrogen source, the use of ammonium salts and urea cannot be excluded from the broad range of possible nitrogen sources. Nevertheless, the excessive use of ammonium salts or urea could damage the cell membrane. Thus, a study on the effect of these nitrogen sources for growth and production of GLA could lead to the selection of better variables among the available nitrogen sources.

Experiments with various nitrogen sources revealed that $NaNO_3$ (0.25%) and $(NH_4)_2SO_4$ (0.01%) affected maximum GLA production (Fig. 2). In the presence of sodium nitrate (0.25% w/v), the algae produced maximum GLA (13.5 mg L^{-1}) and GLA-rich total fatty acid (23% TFA). Ammonium sulfate at a lower concentration (0.01%) showed a considerable effect on GLA yield (11.89 mg L^{-1}) and composition (18% TFA). However, potassium nitrate and urea produced a smaller quantity of fatty acid. Urea at a concentration of 0.01% stimulated a high biomass (1.65 g L^{-1}). Because of its low specific GLA, the algae produced a smaller GLA yield (6.4 mg L^{-1}). Cultivations at higher concentrations of ammonium sulfate (0.1%) and urea (0.1%) showed the complete inhibition of algal growth (data not shown).

In the context of the above observations, various nitrogen sources affecting GLA production were ranked in the following order: $NaNO_3$ >$(NH_4)_2SO_4$>KNO_3>Urea. Earlier findings [9] reported good growth and GLA with sodium nitrate and potassium nitrate at various levels (0.01, 0.03 and 0.05 M) and ammonium salt concentration (0.01 M). These studies also reported urea at 0.01 M, and they supported a GLA yield of 20.8 μg mL^{-1} and 13.5 mg/g GLA. These findings showed that nitrates are the best choice for targeting a quality biomass for GLA production.

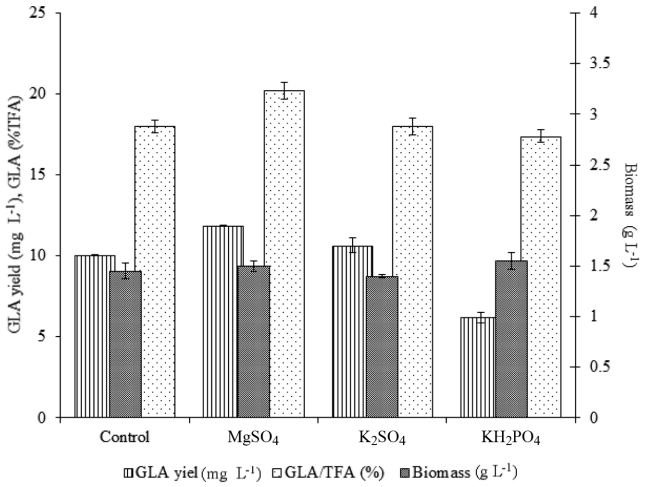

Fig. 3. Effect of mineral sources on biomass, GLA yield and composition. Minerals effecting GLA Yield, which have shown statistically significant difference in the means were selected. One way annova: Between groups (F=38.95; P<0.05).

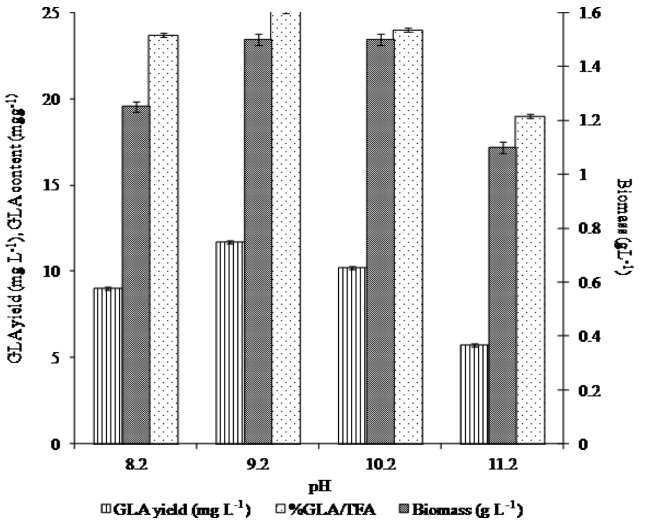

Fig. 4. Effect of pH on algal growth and GLA production. Statistically significant means (GLA yield) was found between various groups (pH in the medium). One way Anova between groups: F=92.2; P<0.05.

3. Effect of Mineral Sources

A typical nutrient analysis of algal biomass shows 914 mg of phosphorus, 257 mg of magnesium, 1.77 g of potassium and 1.05 g of sodium per 100 g biomass [24]. The above analysis showed that minerals play a vital role in the growth and subsequent accumulation of minerals in the algal biomass.

Among the mineral sources studied (Fig. 3), magnesium sulfate hepta-hydrate and potassium sulfate showed better results over control grown cultures. Although elevated levels of ortho-phosphate (6 mM) in the medium supported growth (1.55 g L^{-1}), the alga accumulated lower specific GLA (g GLA/g biomass), which resulted in poor GLA yield (6.2 mg L^{-1}). The algal strain accumulated higher GLA with magnesium salt (11.85 mg L^{-1}) and potassium (10.64 mg L^{-1}) than in the presence orthophosphate. Interestingly, no significant change in growth was observed at 6 mM mineral concentration. Mahajan and Kamat [9] reported maximum specific GLA (10.13 mg g^{-1}) and GLA/TFA (20.9%) at 0.07% (w/v) K_2HPO_4. However, the present results showed that the scope for improvement of GLA production is increased with both MgSO4.7H_2O and K_2SO_4. Thus, the results showed that various mineral salts affecting GLA production could be ordered as follows: Mg $SO_4 \cdot 7H_2O$>K_2SO_4>K_2HPO_4.

4. Effect of Initial pH

Although *Spirulina* cultures can tolerate a wide range of pH conditions, high pH is mandatory for their growth. The optimum pH range is between 8.2-10.0 [25]. *Spirulina* cultures require a good buffering system, which is effectively provided by the bicarbonate in the medium.

In shake-flask studies, little difference was noticed between the initial and final pH values after cultivating the alga for eight days in SOT medium. The biomass (1.5 g L^{-1}), GLA yield (11.7 mg L^{-1}) and GLA to fatty acid ratio (25.1%) were highest at initial pH 9.2 (Fig. 4). It can also be inferred that the cell growth and fatty acid production were stable in the pH range of 9.2-10.2; however, fatty acid production seemed to be affected above and below the optimum pH level.

The influence of pH on algal growth and fatty acid production could be attributed to the relative abundance of bicarbonate at the optimal pH level. Because bicarbonate co-exists in equilibrium with CO_2 (acidic pH) and CO_3^{2-} ion (pH: 11.0), fine shifts in medium pH alter the carbon source available to the algae. The relative abundance of carbon in ionic form controls the specific growth rate and thus the specific production rate of the fatty acid. In the present study, the algae grew well in bicarbonate-enriched medium at alkaline pH (9.2), which showed that it was an optimum pH for GLA production.

5. Orthogonal Array Experiments

In a series of experiments using the OFAT method, medium com-

Table 1. Various factors and their concentration levels selected for the orthogonal array experiments

Level	A (g L^{-1})	B (g L^{-1})	C (g L^{-1})	D (g L^{-1})
1	10	1.0	0.1	1.0
2	20	3.0	0.5	1.5
3	30	9.0	1.0	2.0

A: $NaHCO_3$; B: $NaNO_3$; C: $MgSO_4 \cdot 7H_2O$; D: KH_2PO_4

Table 2. Different factors (media components) and their levels affecting GLA production in orthogonal array runs

Run S. No	Media components				Response	
	A	B	C	D	Biomass (g L^{-1})	GLA (mg L^{-1})
1	1	1	1	1	1.3±0.04	4.87±0.14
2	1	2	2	2	1.8±0.13	11.42±0.36
3	1	3	3	3	2.0±0.1	3.61±0.35
4	2	1	2	3	1.9±0.03	17.73±0.58
5	2	2	3	1	2.0±0.1	11.10±0.19
6	2	3	1	2	2.2±0.17	12.22±0.33
7	3	1	3	2	1.9±0.03	6.37±0.15
8	3	2	1	3	2.2±0.1	8.39±0.64
9	3	3	2	1	2.4±0.3	9.23±0.32

Table 3. The response in terms of means for GLA yield for various factors in the experimental runs

Level	A	B	C	D
1	6.63	9.65	8.49	8.4
2	13.68	10.30	12.79	10.0
3	7.99	8.35	7.02	9.9
Delta	7.05	1.95	5.76	1.60

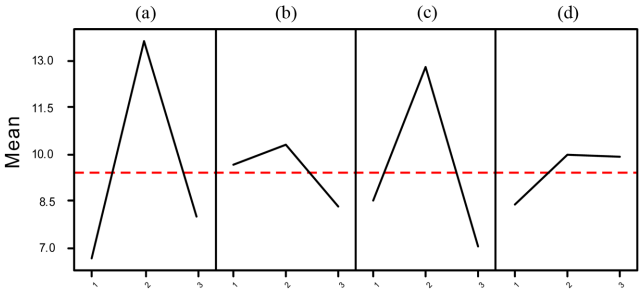

Fig. 5. Main effect plot for GLA production by *Spirulina platensis* with various concentrations of $NaHCO_3$ (a); $NaNO_3$ (b); $MgSO_4$ (c) and K_2SO_4 (d).

ponents showing greater variation in GLA production were selected for orthogonal experiments. Thus, four factors at three concentration levels (Table 1) were considered for the final orthogonal array. In the experimental runs (Table 2), the effect of each factor level (medium component) in a given combination was studied in terms of response (biomass and GLA yield). The data provided in Table 2 show that the fourth experimental combination gave a maximum response in terms of GLA yield (17.73 mg L^{-1}), with a corresponding biomass concentration of 1.97 g L^{-1}. The Minitab, using the data (GLA yield) from the orthogonal array runs, generated a response table (Table 3) of means (the larger the better). The main effect plot (Fig. 5) is a graphical representation of the average values of the means shown in the response table (Table 3).

6. Analysis of Taguchi Results and Prediction of Yields

The response table of means (Table 3) shows the average means (GLA yield) for each media component (A, B, C, and D). As both Table 2 and Fig. 5 show, the factors were the best at the following levels: A (2), B (2), C (2), and D (2). The delta shown in Table 3 is the difference between the highest and the lowest average value of each factor. Various factors were assigned ranks based on the delta values. In the present experiment, the factors affecting GLA production were ranked according to their delta values in the following order of priority: $NaHCO_3$>$MgSO_4 \cdot 7H_2O$>$NaNO_3$>K_2SO_4.

The plots shown in the response table (Table 3) and the main effect show that the factor with the highest impact on the means was $NaHCO_3$. Because it showed the best input levels of media components, Minitab predicted the possible yield in terms of means. In the present experiment, with the new input levels of medium components (A: 2; B: 2; C: 2 and D: 2), the Minitab predicted a theoretical GLA yield of 18.47 mg L^{-1}. The predicted yield, followed by an experimental run (Fig. 6) with the new combination of medium components, produced 19.2±0.4 mg L^{-1} GLA. Thus, the results of the new combination correlated with the theoretical value.

Kim et al. [26] reported the highest GLA content at 2% (*w/w*)

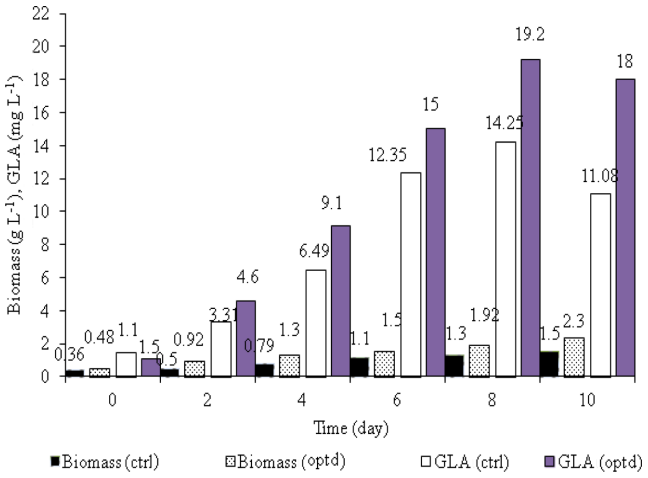

Fig. 6. Variation of biomass and GLA concentration in optimized (Optd) and control (Ctrl) medium. The maximum GLA yield in control and optimized medium were 14.25±0.5 and 19.2 ±0.4 respectively.

and a productivity of 27.6±4.7 mg L^{-1} in *S. platensis*. However, this result was made possible by externally supplementing 0.01 mM palmitic acid in the two-stage culture. Similarly, Mahajan and Kamat [9] reported 20.8 μg mL^{-1} at 0.01 M urea. The final medium after optimization, which comprised different components, had the following composition (g L^{-1}): $NaHCO_3$: 20.0; $NaNO_3$: 3.0; $MgSO_4 \cdot 7H_2O$: 0.5; K_2SO_4: 1.5; K_2HPO_4: 0.5; NaCl: 1.0; $FeSO_4 \cdot 7H_2O$: 0.01; $CaCl_2 \cdot 2H_2O$: 0.04; EDTA: 0.08; and 1 mL of A5 trace metal solution with medium pH 9.2.

CONCLUSION

Optimizing media components using orthogonal array experiments is a preferable approach for experiments involving a large number of variables. Based on experiments using the one factor method, medium components ($NaHCO_3$; $MgSO_4 \cdot 7H_2O$; $NaNO_3$; K_2SO_4) showing greatest variation in GLA production were selected for additional experiments. The orthogonal experiments demonstrated increased GLA production. In this study, the improvised concentration levels of medium components showed a 1.4-fold increase in GLA production (13.5 mgL^{-1} at 0.25% $NaNO_3$ versus 19.2 mgL^{-1} at 0.3% $NaNO_3$), compared with the original inorganic SOT medium. Since medium components are one of several other factors that influence algal growth and fatty acid production, our results indicate a greater scope for further increases in GLA yield by cultivating algae in this medium under optimal conditions.

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